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THE PROBLEM OF IMMUNIZATION AGAINST STAPHYLOCOCCAL MASTITIS

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INTRODUCTION

The possibility of immunizing the dairy cow against mastitis has claimed the attention of research workers for most of this century. In his survey of the literature to the end of 1935 Munch-Petersen (1938) listed 87 reports of attempts to immunize cattle against mastitis. The success of these and subsequent attempts has been qualified (Mellenberger, 1977) and currently the attitude is of exasperated optimism: the cow should be able to mount a significant immune response in the mammary gland if only an effective antigen is given by the correct route at the proper stage in the lactation cycle (Pankey, 1977).

The purpose of this paper is not to review the numerous attempts that have been made to immunize against staphylococcal mastitis, but to bring together the knowledge of the way in which staphylococci cause infection and the way in which the mammary gland responds to the presence of staphylococci, in order to identify and evaluate the problems inherent in immunization.

THE DEFENCE SYSTEM

The premise is that the principal mechanism by which the lactating mammary gland removes staphylococci that have entered the mammary tissue is by phagocytic killing by neutrophils. A virulent staphylococcus is one that resists phagocytic killing and though the way in which this occurs is not clear (Anderson, 1976), it remains true that phagocytic killing by neutrophils is the method of host defence. Successful phagocytosis involves recruitment of neutrophils, opsonization and killing.

Recruitment of neutrophils

Neutrophils accumulate in the alveolar lumen of the mammary gland by recruitment from the systemic circulation and this occurs in response to the generation of chemotactic factors by the staphylococci. Chemotactic factors may act directly on the corresponding cell (cytotaxins) or may, by acting on enzyme systems or complement, cause the release of chemotactic peptides (cytotaxigens) (Keller & Sorkin, 1967). In addition, complement activation results in the formation of anaphylotoxins which, through histamine release, increase the permeability of blood vessels. The

products of staphylococcal growth have been shown to possess little direct chemotactic activity and some inhibition of neutrophil locomotion. However indirect chemotactic substances are generated by the combination of staphylococcal growth products and plasma components (Russell *et al.*, 1976). Although these actions have been demonstrated only *in vitro* it is tempting to suggest that *in vivo* the cytotaxigenic factors attract neutrophils to the site of staphylococcal infection and that the inhibitory activity immobilizes the neutrophils in the vicinity of the staphylococci.

Opsonization

Ingestion of staphylococci by neutrophils is facilitated by opsonization of the organisms. The first of the two major systems in serum which are opsonic for staphylococci is heat-labile (56°C for 30 min) and involves the activation of complement through the alternate (properdin) or classical (IgG or IgM) pathways so that the opsonically active fragments of complement are deposited on the surface of staphylococci and bind them to neutrophils. The other system is heat-stable and involves the attachment of IgG to the organisms through the Fab fragment; there are receptors for the Fc portion of the antibody molecule on the surface of the neutrophil so that recognition and ingestion are promoted (Winkelstein, 1973; Stossel, 1974a).

Apart from the first few days after parturition in the cow when the colostrum is rich in IgG, IgM and IgA, the predominant immunoglobulin in normal milk is IgG1 (Butler, 1973). The transfer of IgG1 into the milk is a selective process which is a function of the glandular epithelium (Brandon, Watson & Lascelles, 1971), the complement content of normal milk is variable (Reiter & Brock, 1975). Thus there are limitations on the antibody and complement content of normal milk. However if the integrity of the epithelium is broken, as by inflammation, then there is a transudation of serum proteins and therefore of immunoglobulins and complement into milk.

Ingestion and killing

During ingestion the cell membrane of the neutrophil invaginates at the point of contact with the opsonized staphylococcus to form a phagosome and the cytoplasmic granules begin to fuse their membranes with that of the phagosome to form a phagolysosome. The secondary granules discharge their lactoferrin and lysozyme into the phagolysosome. Myeloperoxidase is discharged from the primary granules and together with H_2O_2, provided by the respiratory burst associated with ingestion, and Cl^-, completes the bactericidal myeloperoxidase-hydrogen peroxide halide system. Lysozyme, elastase and cationic proteins are also derived from the primary granules and function as oxygen independent bactericidal systems. There are therefore a number of bactericidal systems in the neutrophil that can act separately or in concert (Stossel, 1974b; Spitznagel, 1977). Once a neutrophil has degranulated no new granules are formed, and though the life-span of neutrophils once they have entered the milk is not known with certainty, it seems likely that it is no more than a few hours. The cells with their ingested material degenerate and are removed by macrophages or ... in the udder. ... ell ingest and kill fewer

1975), but is due to the presence in milk of casein micelles and fat globules which are ingested by neutrophils and exhaust the cells by diverting the discharge of granules into phagolysosomes that contain casein or fat (Paape *et al.*, 1975; Russell, Brooker & Reiter, 1976). It is probable that this abortive discharge of granules occurs *in vivo* but the detrimental effect of the phenomenon may be overstated. The short life-span of neutrophils in the udder means that these cells can only be efficient for a few hours after extravasation. Since newly extravasated neutrophils are as efficient as circulating neutrophils in their bactericidal capacity the studies with casein and fat primarily indicate that efficient neutrophil phagocytosis requires a constant flow of new mature neutrophils into the alveolar lumen. The studies with casein have also shown that there is no defect in degranulation following ingestion of staphylococci (Russell, Brooker & Reiter, 1977). Degranulation occurs normally. It appears to be characteristic of virulent staphylococci that they survive in phagosomes into which the contents of granules have been discharged.

The consequences of ingestion of staphylococci by a neutrophil are illustrated in Fig. 1.

IMMUNIZATION

Protection by immunization is always achieved by utilizing and enhancing the natural method by which a host would defend itself against disease. An understanding of the way in which a disease is produced and knowledge of the host defence mechanisms are therefore prerequisites of any rational attempt at immunization. For example, since tetanus is caused by a neurotoxin produced by growth of *Cl. tetani* in an area of tissue in which there is a reduced oxygen potential, immunization can be effected by presenting the toxin to the host in a non-toxic but immunogenic form so that antibodies are made against the toxin and are immediately available to neutralize the toxin should it be produced in the host. The method by which the mammary gland defends itself against staphylococci has been outlined: effective immunization can operate only by enhancing some stage in the natural mechanism of host defence.

There is, however, a constraint. In the development of a definition of mastitis stress has been increasingly laid on the presence of potentially pathogenic bacteria and elevated milk somatic cell counts in asceptically sampled foremilk, often in the absence of clinical signs. Thus the International Dairy Federation says 'in all these definitions (normal udders, latent infection, subclinical, clinical and chronic mastitis) the cell count of the milk is the most important criterion, as the threshold value of normal foremilk is recognized as being no higher than 500 000 cells/ml' (Ann. Bull., 1971). This is not an arbitrary figure but is the upper value of two standard deviations about a mean cell count which was the point at which rising cell count coincided with deviations from normal values of sodium, potassium, chloride, lactose and whey nitrogen in milk and of milk yield, and which indicated secretory disturbances in the mammary parenchyma (Reichmuth, 1975). While there may be objections to the emphasis laid on somatic cell counts in diagnosing mastitis in individual cows (Giesecke & van den Heever, 1974) the value of bulk-milk somatic cell counts, despite their limitations (Wright, 1977), should not be underestimated in monitoring a mastitis control programme.

However, if mastitis is 'a condition of the mammary gland such that there has been an evocation of the cytological defence mechanism with a consequent rise in somatic

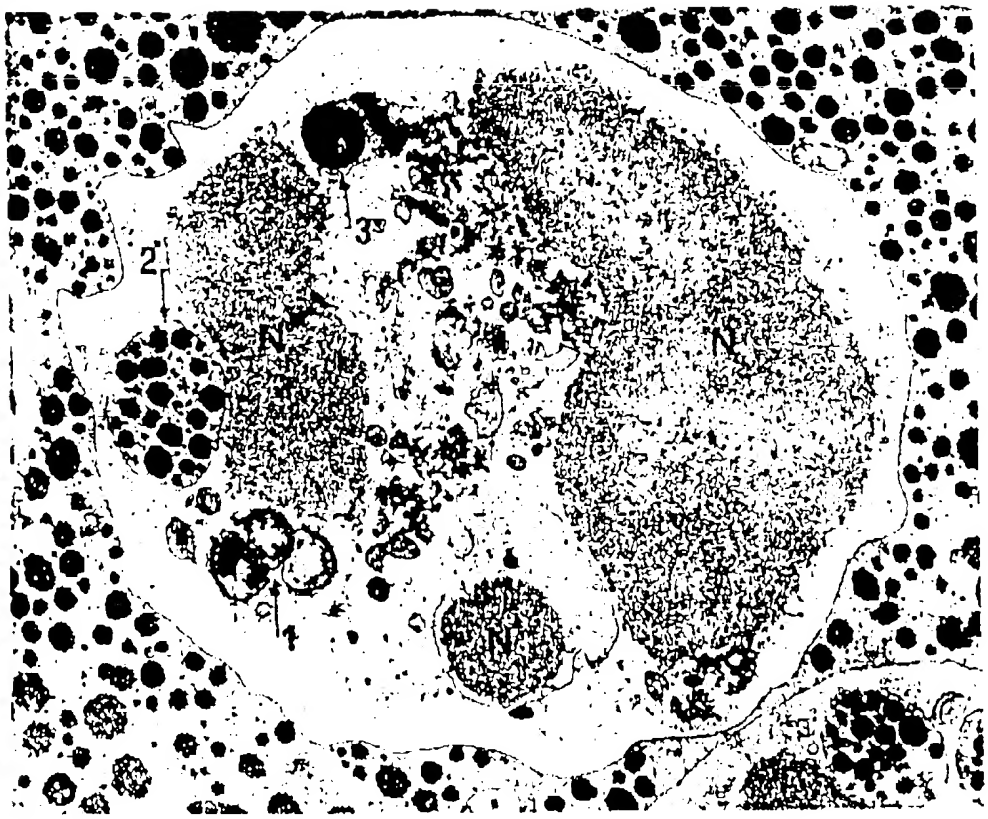

Fig. 1. Neutrophil from a mouse mammary gland inoculated with *Staphylococcus aureus*. There is a group of three staphylococci undergoing destruction in a phagolysosome (1). There is a phagosome full of casein (2) and a phagolysosome containing a normal staphylococcus (3). The neutrophil has degranulated and there are early degenerative changes in the nucleus (N). (x 14 600.)

cell levels' (Reichmuth, Tolle & Whittlestone, 1976), then it becomes evident that the natural defence mechanism of the mammary gland cannot be used, whether or not enhanced by immunization, to prevent mastitis since it is the defence system that is defined as the disease. Immunization, which cannot operate other than through the natural defence system, must of necessity function, when the mammary gland is challenged by staphylococci, by creating the response (mastitis) it is directed to prevent.

The problem is not removed by redefining mastitis; the destinction between 'infection' and 'inflammation' of the udder is not at issue. The emphasis on somatic cell numbers in the definition of mastitis has merely high-lighted the problem. The only inflammatory response under consideration is that due to invasion of the mammary gland by staphylococci. Prasad & Newbould (1968) showed that when small numbers of staphylococci were infused into the udder inflammation invariably resulted and, following infusion of staphylococci, there was never inflammation without infection (Newbould & Neave, 1965). If mastitis is defined only in terms of isolation of staphylococci (Neave, 1975) then the defence system is no longer defined as the disease, but an inflammatory response and isolation of bacteria are so inextricably bound that the argument still holds that challenge of the mammary gland of immunized animals must result in mastitis.

Current definitions of mastitis, whether in terms of somatic cell numbers (Ann. Bull. 1971), bacterial isolation (Griffin *et al.*, 1977) or a combination of both (Pearson & Greer, 1974), indicate the high standards demanded of normal milk and which immunization must satisfy to be successful.

POSSIBLE TYPES OF IMMUNITY

There are five stages in the defence system that might be enhanced by immunization.

1. *Neutrophil recruitment*

Since recruitment of neutrophils is dependent upon the elaboration of chemotactic factors by multiplying staphylococci there is a delay between the entry of the organisms into the gland and the arrival of neutrophils. Targowski & Berman (1975) observed that the leucocyte response to a first intramammary infusion of staphylococcal antigens in lactating cows, previously parenterally vaccinated with killed cells in oil-water adjuvant, was comparable to the leucocyte response to a second intramammary infusion in non-vaccinated cows. This increased and more rapid neutrophil response in sensitized cows was interpreted as an expression of cell mediated immunity and suggests a method by which neutrophil recruitment may be accelerated. It has also been argued that activation of the reticulo-endothelial system by staphylococcal antigens should be beneficial in defence against infection (Jones, 1974). Jones recommends the elicitation of cell-mediated immunity in the dry period since macrophages are more numerous in the involuted udder. However an immunologically elicited neutrophil response in lactating cows, though it may succeed in limiting the development of infection, is nevertheless mastitis. In addition, cell-mediated immunity *per se* can be harmful rather than beneficial as has been elegantly [illegible] & Gilmour, 1975) and both

Targowski & Berman (1975) and Jones (1974) are guarded in their recommendation of this form of therapy.

2. *Anti-toxin immunity*

Toxins, especially alpha toxin, play an important part in staphylococcal mastitis and it would seem logical to incorporate them in toxoided form in a vaccine. However, our understanding of the role of alpha toxin is that its production is a terminal event in the pathogenesis of the disease (Anderson, 1976). Alpha toxin is produced and released only towards the end of logarithmic bacterial growth in the mammary gland, that is, at a time when mastitis already exists. Anti-alpha toxin neutralizes alpha toxin; it can therefore only ameliorate the terminal pathological changes in the disease and can have little or no effect on the establishment and early development of mastitis. This view is supported by the observation that in older cows, that have higher levels of serum anti-alpha toxin, the incidence of acute clinical episodes is lower (Schalm, Carroll & Jain, 1971).

Anti-alpha toxin is present in normal milk in lower concentration than in the blood (Edwards & Smith, 1959) and it reaches the milk in the early stages of disease only when the epithelial barrier is damaged by the products of bacterial multiplication (Symons & Wright, 1974). This results in high levels of anti-toxin in milk (Derbyshire, 1960a), but since the epithelium is breached there is also a neutrophil infiltration, and so mastitis exists in terms of high somatic cell count and of staphylococcal excretion.

Although complicated by the inclusion of cell walls with alpha toxin, the vaccination experiments of Derbyshire (1960b) illustrate the problem of immunization in relation to anti-toxin immunity. When non-vaccinated cows were challenged with staphylococci a gangrenous mastitis with complete loss of secretion developed whereas only a mild transient reaction occurred in the vaccinated animals. It is probable that the anti-alpha toxin was responsible for abrogating the gangrenous reaction in the vaccinated cows. Nevertheless, because of the elevated neutrophil response and the continued excretion of staphylococci, the reaction in these vaccinated cows must be classified as mastitis. When rabbits that were immunized with highly purified alpha toxin were challenged with *Staph. aureus* it was found that they were protected against the lethal haemorrhagic oedematous form of the disease but they were not protected against the chronic abscess form (Adlam *et al.*, 1977). Conversely, when goats that were vaccinated with a preparation that did not contain alpha toxin were challenged with staphylococci, severe clinical and gangrenous reactions were obtained (Lepper, 1967). Alpha toxin should be incorporated in any vaccine against staphylococcal mastitis, but it cannot rationally be expected to do any more than prevent acute clinical flare-ups, and can have no effect on chronic or subclinical infections.

3. *Anti-bacteria immunity*

Bactericidal substances exist in milk in the form of lactoferrin and the lacto-peroxidase-thiocyanate-hydrogen peroxide system but, though these systems are active against staphylococci (Reiter & Bramley, 1975), they are not antibodies and therefore outside the influence of immunization. Similarly, the substances in serum which are bacteristatic for staphylococci are generally considered to be non-antibody in nature, though it has been suggested that natural antibody is involved (Ehrenkranz,

Elliott & Zarco, 1971). The antibacterial activity of serum is inhibited by coagulase thus strongly coagulase positive strains of staphylococcus grow luxuriously in serum (Ekstedt, 1956).

As regards specific antibody, it is generally accepted that Gram-positive organisms are not readily killed by the complement mediated bactericidal activity of antibodies; both antibody or complement and neutrophils are required. It seems unlikely, therefore, that a protective mechanism can be developed which depended only on specific antibacterial antibody, even if that antibody could be transported across an intact secretory epithelium.

4. *Anti-enzyme immunity*

The extracellular proteins of staphylococci, many of which have enzymic properties, have been implicated in the pathogenesis of staphylococcal mastitis (Anderson, 1976). Knowledge of the properties of coagulase suggests that it should be one of the most promising of antigens. It is produced early in the logarithmic phase of growth and if it is essential for growth of staphylococci *in vivo* its neutralization at this stage might render staphylococci more susceptible to non-antibody bactericidal substances, and so prevent the establishment of infection. Antibodies can be raised against purified coagulase (Blobel, Berman & Simon, 1960) but in order to protect the mammary gland the antibody would require to lie in IgG1 so that it would reach the milk without a simultaneous neutrophil response.

No record seems to exist of an attempt to immunize against mastitis using only coagulase as antigen. Coagulase was incorporated in a vaccine that was tested in goats but Derbyshire and Helliwell (1962) were unable to attribute any protective effect to that antigen. A vaccine which contained coagulase was tested in cattle and, though the criteria on which mastitis was diagnosed were not clearly stated, a reduction in the incidence and clinical severity of mastitis caused by the homologous strain was recorded (Blobel & Berman, 1962). There was no protective effect against heterologous strains of staphylococci.

5. *Opsonin immunity*

The available evidence indicates that staphylococci are killed in the mammary gland most effectively by the opsonin-neutrophil system. Detailed studies in the mammary gland of the mouse (Anderson & Chandler, 1976) and *in vitro* studies with bovine leucocytes (Russell *et al.*, 1977) show that there is no defect in ingestion of staphylococci by neutrophils but that degranulation occurs without killing of ingested staphylococci. To be effective immunization must elicit antibodies which not only facilitate ingestion but also render the staphylococci susceptible to the action of the contents of the neutrophil granules.

Two recent attempts to achieve such an immunity have been reported. Watson (1975) showed *in vitro* that neutrophils from ewes that had been infected with staphylococci as well as inoculated with killed staphylococci were more phagocytic for staphylococci than neutrophils from ewes that had received only the killed staphylococci. The enhanced phagocytosis (killing) was later shown to be due to a

responsible for the cytophilic antibody and whether the purified antigen will be sufficiently immunogenic. However, to function *in vivo* IgG2, which could only enter the alveolar lumen in large quantity by transudation since it is not the predominant antibody to be selectively transferred across the epithelium, together with neutrophils would require to accumulate in the alveolar lumen in response to replicating staphylococci. That is, immunization would work by evoking mastitis, albeit enhanced mastitis.

Brock, Turvey & Reiter (1973) showed that if *Staph. aureus* was grown in a high salt-high carbohydrate medium analogous to milk its virulence was increased because of the production of extracellular slime. It is possible that the slime acts as a pseudo-capsule and makes some strains more resistant to killing by neutrophils. Antibody to this extracellular slime should therefore enhance the opsonin-neutrophil system. However, when cows were injected with dead staphylococci prepared from high salt-high carbohydrate medium and challenged, there was no evidence of resistance to infection (Brock, Steel & Reiter, 1975). Vaccination did not result in higher levels of immunoglobulin in serum, colostrum or milk as compared to controls despite a rigorous vaccination schedule that involved Freund's adjuvant and intramammary as well as intramuscular injections. In the study 'infection' was not clearly defined but the neutrophil response and the excretion of staphylococci in the cows that were vaccinated and challenged was presumably sufficient to constitute mastitis.

The problem of immunization against staphylococcal mastitis is illustrated in the detailed study by Singleton *et al.* (1967). Teichoic acid was justified as antigen and goats received three doses of antigen with adjuvant by the subcutaneous route. Three of the six challenged vaccinated goats were said to be immune. However, examination of the data for these three goats shows that following challenge the number of neutrophils in the milk was high and that staphylococci were excreted in the milk for at least a week. Therefore by current standards these 'immune' goats must be deemed to have mastitis.

CONCLUSION

It is normal to cite the disparity of antigenic types, the need to identify virulence factors and make effective antigenic preparations, and the lack of a proper inoculation route and time schedule in relation to the lactation cycle as among the obstacles to immunization against staphylococcal mastitis in dairy cows. These difficulties together with the fact that mastitis can be caused by several bacterial species other than *Staph. aureus*, may be themselves sufficient to prevent the formulation of an effective and acceptable vaccine against mastitis. But there is a more fundamental problem which is particularly relevant to immunization against staphylococcal mastitis, namely, that the invasion of the mammary gland by pathogenic staphylococci results in an inflammatory response which as a milk somatic cell count together with excretion of staphylococci are criteria of mastitis. But the inflammatory response is the natural mechanism by which the mammary gland defends itself against staphylococci and immunization can only function by enhancing this mechanism. It is therefore inevitable that the reaction in the immunized gland to invasion by staphylococci will constitute mastitis. Only when protection can be achieved by eliciting an inflammatory

response which is insufficient to constitute subclinical mastitis, will immunization against bovine mastitis have succeeded.

REFERENCES

ADLAM, C., WARD, P. D., McCARTNEY, A. C., ARBUTHNOTT, J. P. & THORLEY, C. M. (1977). *Infec. Immunity* 17, 250.

ANDERSON, J. C. (1976). *Br. vet. J.* 132, 229.

ANDERSON, J. C. & CHANDLER, R. L. (1975). *J. comp. Path.* 85, 499.

ANNUAL BULLETIN (1971). *Int. Dairy Fed.* Part 1.

BLOBEL, H. & BERMAN, D. T. (1962). *Am. J. vet. Res.* 23, 7.

BLOBEL, H., BERMAN, D. T. & SIMON, J. (1960). *J. Bact.* 79, 807.

BRANDON, M. R., WATSON, D. L. & LASCELLES, A. K. (1971). *Aust. J. exp. Biol. med. Sci.* 49, 613.

BROCK, J. H., STEEL, E. D. & REITER, B. (1975). *Res. vet. Sci.* 19, 152.

BROCK, J. H., TURVEY, A. & REITER, B. (1973). *Infec. Immunity* 7, 865.

BUTLER, J. E. (1973). *J. Am. vet. med. Ass.* 163, 795.

DERBYSHIRE, J. B. (1960a). *Res. vet. Sci.* 1, 350.

DERBYSHIRE, J. B. (1960b). *J. comp. Path. Ther.* 70, 222.

DERBYSHIRE, J. B. & HELLIWELL, B. I. (1962). *Res. vet. Sci.* 3, 56.

EASMON, C. S. F. & GLYNN, A. A. (1975). *Immunology* 29, 75.

EDWARDS, S. J. & SMITH, C. S. (1959). *J. comp. Path. Ther.* 69, 208.

EHRENKRANZ, N. J., ELLIOTT, D. F. & ZARCO, R. (1971). *Infec. Immunity* 3, 664.

EKSTEDT, R. D. (1956). *J. Bact.* 72, 157.

GIESECKE, W. H. & VAN DEN HEEVER, L. W. (1974). *Onderstepoort J. vet. Res.* 41, 169.

GRIFFIN, T. K., DODD, F. H., NEAVE, F. K., WESTGARTH, D. R., KINGWILL, R. G. & WILSON, C. D. (1977). *J. Dairy Res.* 44, 25.

JONES, R. L. (1974). *Proc. Ann. Meet. U.S. Anim. Hlth Ass.* 78, 143.

KELLER, H. U. & SORKIN, E. (1967). *Int. Archs Allergy appl. Immun.* 31, 505.

LEPPER, A. W. D. (1967). *Br. vet. J.* 123, 183.

MELLENBERGER, R. W. (1977). *J. Dairy Sci.* 60, 1016.

MUNCH-PETERSEN, E. (1938). *Imp. Bur. Anim. Hlth, Rev. Ser.* No. 1.

NEAVE, F. K. (1975). *Proc. Seminar on Mastitis Control, Int. Dairy Fed.* 85, 19.

NEWBOULD, F. H. S. & NEAVE, F. K. (1965). *J. Dairy Res.* 32, 163.

PAAPE, M. J., GUIDRY, A. J., KIRK, S. T. & BOLT, D. J. (1975). *Am. J. vet. Res.* 36, 1737.

PANKEY, J. W. (1977). *J. Am. vet. med. Ass.* 170, 1231.

PEARSON, J. K. L. & GREER, D. O. (1974). *Vet. Rec.* 95, 252.

PRASAD, L. B. M. & NEWBOULD, F. H. S. (1968). *Can. vet. J.* 9, 170.

REICHMUTH, J. (1975). *Proc. Seminar on Mastitis Control, Int. Dairy Fed.* 85, 93.

REICHMUTH, J., TOLLE, A. & WHITTLESTONE, W. G. (1976). *N.Z. vet. J.* 24, 47.

REITER, B. & BRAMLEY, A. J. (1975). *Proc. Seminar on Mastitis Control, Int. Dairy Fed.* 85, 210.

REITER, B. & BROCK, J. H. (1975). *Immunology* 28, 71.

RUSSELL, M. W. & REITER, B. (1975). *J. Reticuloendothel. Soc.* 18, 1.

RUSSELL, M. W., BROOKER, B. E. & REITER, B. (1976). *Res. vet. Sci.* 20, 30.

RUSSELL, M. W., BROOKER, B. E. & REITER, B. (1977). *J. comp. Path.* 87, 43.

RUSSELL, R. J., WILKINSON, P. C., McINROY, R. J., McKAY, S., McCARTNEY, A. C. & ARBUTHNOTT, J. P. (1976). *J. med. Microbiol.* 9, 433.

SCHALM, O. W., CARROLL, E. J. & JAIN, N. C. (1971). In *Bovine Mastitis.* Philadelphia: Lea & Febiger.

SINGLETON, L., ROSS, G. W., STEDMAN, R. A. & CHANTER, K. V. (1967). *J. comp. Path.* 77, 279.

[illegible] *The Granulocyte: Function and Clinical Utilization*, p. 103, New York:

SIMONS, D. B. A. & WRIGHT, L. J. (1974). *J. comp. Path.* **84**, 9.
TARGOWSKI, S. P. & BERMAN, D. T. (1975). *Int. J. vet. Res.* **36**, 1561.
WATSON, D. L. (1975). *Res. vet. Sci.* **19**, 283.
WATSON, D. L. (1976). *Immunology* **31**, 159.
WINKELSTEIN, J. A. (1973). *J. Pediat.* **82**, 747.
WRIGHT, C. L. (1977). *Vet. Rec.* **100**, 8.

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